THE RESPIRATION OF UNFERTILIZED SEA URCHIN EGGS IN THE PRESENCE OF ANTISERA AGAINST FERTILIZIN [1]

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It has been shown (Tyler and Brookbank, 1956a, 1956b) that antisera against purified fertilizin, as well as antisera against homogenates of fertilized and unfertilized eggs deprived of the gelatinous coat material, increase the respiratory rate of demembranated fertilized eggs, and cause an inhibition of cleavage. The respiratory rate of blocked eggs increases to a maximum of 4 to 5 times the control rate in 20 to 40 minutes, and subsequently decreases to the rate of the controls during the next 40 minutes. Gradual cytolysis of the eggs occurs following 4–5 hours of exposure to undiluted antisera. The observed increase in the respiratory rate in the presence of immune serum is apparently without parallel in the previous literature. Haurowitz and Schwerin (1940) studied the respiration of pigeon erythrocytes in the presence of immune rabbit serum and active and inactive complement, yielding lysis and agglutination, respectively. No increase or decrease in respiratory rate was noted in either case. Sevag and Miller (1948), studying the respiration of *E. typhosa* (strain 0–901) in the presence of immune rabbit serum and active or inactive complement, found that intact, sensitized cells consumed oxygen at the same rate as the controls. However, in the presence of active guinea pig complement, the cells lysed, with an accompanying transitory increase (1.4-fold), which was followed by a decrease to one-fourth the control rate after 180 minutes. Harris (1948) measured the oxygen uptake of Salmonella cells in the presence of agglutinating rabbit serum, and found no increase or decrease in respiratory rate over a wide range of antiserum concentrations. Nowinski (1948) investigated the possibility of an effect on respiratory rate by reticulo-endothelial-immune-serum (REIS) acting on rat spleen slices, and by anti-chick brain serum acting on chick brain homogenates (1949). No effect on respiration was observed with REIS, and a slight inhibition of oxygen uptake of chick brain homogenates was noted in the presence of anti-chick brain serum. MacDonald (1949) obtained similar results with REIS using rat spleen slices in Thunberg experiments.

The purpose of the present experiments was to explore the effect of antisera against fertilizin, which is, chemically, a rather well defined substance (Tyler, 1949, 1956), on the respiratory rate of unfertilized sea urchin eggs. Unfertilized eggs, though normally respiring at a low rate, can be stimulated to respire at a much

greater rate (4–5-fold increase) by parthenogenetic agents (Warburg, 1908; Keltch and Clowes, 1947), by nitrophenols (Clowes and Krahl, 1934, 1936) and other non-parthenogenetic substances, as well as by fertilization. In this connection, it is noteworthy that Perlman (1954, 1957), and Perlman and Perlman (1957) have reported that antisera against fertilizin, as well as antisera against extracts of unfertilized eggs, are capable of activating the unfertilized eggs of *Paracentrotus lividus*. The respiratory rate of eggs so treated therefore becomes of interest.

Materials and Methods

Preparation of antigens and antisera

1) *Lytechinus variegatus* (Cedar Key, Florida). Fertilizin antigens were prepared from neutralized supernatant egg-water of acid- (pH 3.5) treated unfertilized eggs. In the case of antigen number 4, the egg-water was dialyzed against distilled water and injected without further purification. Antigen number 10 was prepared by first precipitating the fertilizin of the egg-water (derived from a second spawn of eggs) with 5/4 volumes of cold 95% ethanol (Tyler, 1949). The fertilizin precipitate was then washed thoroughly with additional volumes of cold ethanol, and vacuum-dried. The dried precipitate was dissolved in distilled water, and used for injection. Both antigens had a final agglutination titer of approximately 1/1000 on homologous sperm.

A whole sperm antigen (number 11) was prepared from washed *Lytechinus variegatus* sperm (presumably free of seminal fluid) which were diluted to a 10% suspension by volume with sea water and frozen until used.

All above antigens were stored at $-20°$ C. in one-ml. aliquots. After thawing for use in injections and various tests, the material remaining in each individual aliquot was discarded. This procedure avoided repeated freezing and thawing of the antigens.

Antisera against the above antigens were prepared in rabbits according to an immunization schedule described elsewhere (Tyler and Brookbank, 1956a). Following a control bleeding, totals of 400 μg N (no. 4), 200 μg N (no. 10), and 6000 μg N (no. 11) were injected into the animals over a period of three weeks. The rabbits were bled by cardiac puncture 5 days following the final injection. The antisera were recovered from the retracted clots, and dialyzed thoroughly against sea water at 10° C., and stored at $-20°$ C.

In addition, an antiserum against extract of washed, demembranated, fertilized eggs of *Lytechinus pictus* was available, and was used in a number of experiments. This antiserum had been previously shown to increase the respiration rate of fertilized *L. pictus* eggs (Tyler and Brookbank, 1956b).[3]

2) *Strongylocentrotus purpuratus* (Friday Harbor, Washington). A single fertilizin antigen was prepared by precipitation of the fertilizin from the egg-water of acid- (pH 3.5) treated eggs by NaOH (in the ratio of 40 ml. 1 N NaOH per liter of egg water; Tyler, 1949). The resulting precipitate was dissolved in sea water, following neutralization of the alkali, and dialyzed against distilled water.

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[3] Due to an oversight on the part of the authors, Tyler and Brookbank (1956b) contains an error on page 312, line 6. This line reads correctly if *L. pictus* is substituted for *S. purpuratus*.

TABLE I

The effect of antisera, normal sera, 0.1% sperm suspension, and hypertonic sea water on the respiration of unfertilized Lytechinus variegatus eggs

Experiment no.	Reagent employed	Aver. rate (μl./min./vessel)	Ratios of aver. rates (increased/control)
1	No. 4 normal serum	0.28	
	No. 4 normal serum	0.28	
	Anti-no. 4	0.67 }	2.4
	Anti-no. 4	0.67 }	
	Anti-*L. pictus* fertilized egg extract	0.73 }	2.6
	Anti-*L. pictus* fertilized egg extract	0.73 }	
	Buffered sea water	0.28	
	Buffered sea water	0.28	
	Buffered sea water	0.33	
2	No. 10 normal serum	0.25	
	Anti-no. 11 serum	0.31	
	Anti-no. 10 serum	1.18 }	4.1
	Anti-no. 10 serum	1.13 }	
	Buffered sea water	0.28	
3	No. 4 normal serum	0.33	
	No. 4 normal serum	0.28	
	Anti-no. 4	1.08 }	3.5
	Anti-no. 4	1.03 }	
	0.1% sperm suspension	1.07	3.5
	Buffered sea water	0.33	
	Buffered sea water	0.28	
	Buffered sea water	0.22	
4	No. 5 normal serum	0.30	
	No. 5 normal serum	0.22	
	Anti-*L. pictus* fertilized egg extract	0.92 }	3.4
	Anti-*L. pictus* fertilized egg extract	0.84 }	
	Buffered sea water	0.27	
5*	No. 11 normal serum	0.14	
	Anti-no. 10	0.95	6.7
	Anti-*S. purpuratus* fertilizin (three anti-sera)	0.37	2.6
		0.33	2.3
		0.33	2.3
	Buffered sea water	0.14	
6	0.1% sperm suspension	0.83 }	3.2
	0.1% sperm suspension	0.80 }	
	Hypertonic sea water	0.84 }	3.3
	Hypertonic sea water	0.84 }	
	Buffered sea water	0.25	
7	Anti-no. 10 undiluted	0.32	2.5
	Anti-no. 10 1:1 dilution	0.26	2.0
	Anti-no. 10 1:2 dilution	0.24	1.8
	Anti-no. 10 1:4 dilution	0.20	1.5
	No. 11 normal serum	0.16	
	Buffered sea water	0.10	
	Buffered sea water	0.12	

* In experiment 5, one ml. of 20% egg suspension was used with vessels of 7-ml. capacity, as opposed to 2.3 cc. of egg suspension with vessels of 15-ml. capacity in the other experiments. Five-tenths ml. of antiserum or normal serum was used throughout all experiments. The conditions in experiment 5 duplicate those which obtained during experiments with eggs of *S. purpuratus*.

After dialysis, the fertilizin was precipitated with 5/4 volumes of cold ethanol, washed, vacuum-dried, redissolved in distilled water, and used for injection into each of three rabbits. This antigen had a sperm agglutination titer of approximately 1/500.

Following a control bleeding, the rabbits were injected according to a previously described schedule (Tyler and Brookbank, 1956a) for a period of 2½ weeks, each receiving a total of 250 μg N. The animals were bled by cardiac puncture 4 days following the last injection.

Manometric methods

1) *Lytechinus variegatus.* The effect of the antisera on the respiration of unfertilized eggs, deprived of all soluble fertilizin by acid (pH 3.5) treatment, was followed, at 20° C., in a standard Warburg apparatus using vessels of approximately 15 ml. total capacity ($k_{0_2} = 1.4$–1.5 μl./mm.). The eggs used in these experiments were 90–100% fertilizable following the acid treatment. The settled eggs were diluted to a 20% suspension, on the basis of settled volume, with buffered sea water (0.01 M glycyl-glycine, pH 8.0). The suitability of glycyl-glycine as a sea water buffer, and as a medium for the eggs, has been established by Tyler and Horowitz (1937). The main chamber of the vessel contained 2.3 ml. of egg suspension, the center well 0.2 ml. of 10% NaOH, and the side arm 0.5 ml. of the test solution (antiserum, normal serum, hypertonic sea water, or sperm suspension). After the respiratory rate of the eggs in buffered sea water had been established (usually after 40 minutes), the side arms were tipped and the rate of respiration in the test solution was determined. At the end of each experiment, the eggs were examined microscopically.

2) *Strongylocentrotus purpuratus.* During the two preliminary experiments reported in Table II, the procedure followed was similar to that described above, with the following exceptions: (1) The temperature employed was 18° C., the maximum tolerated by these eggs. (2) One ml. of a 20% suspension, in buffered sea water, was exposed to 0.5 ml. of serum, added from the side arm of vessels of ca. 7-ml. total capacity ($k_{0_2} = 0.7$–0.8 μl./mm.). Thus, the ratio of ml. of serum to the final volume in the vessel was increased, in these experiments, from 0.18 to 0.33 ml. serum/ml. (3) In experiment 2, Table II, the egg suspension was exposed to 1 mg% trypsin solution (crystalline, lyophyllized trypsin—Worthington Biochemical Corp., Freehold, N. J.) for 10 minutes prior to suspension in buffered sea water. This treatment prevented the elevation of the fertilization membrane of test eggs inseminated in sea water following washing. The eggs used in both experiments were 95–100% fertilizable after acid treatment. Trypsin treatment reduced this figure to 30% in experiment 2, using the same amount and concentration of sperm suspension for each test insemination.

Results

As can be seen in Table I, all antisera against fertilizin increased the respiratory rate of unfertilized Lytechinus eggs, including those antisera directed against *S. pupuratus* fertilizin. The increase noted in heterologous antisera was somewhat less than that observed using homologous antisera. Normal sera and antiserum

against sperm effected no measureable increase in respiratory rate compared with the rate observed in buffered sea water. The increased respiration in the presence of antisera against fertilizin reached a maximum at 20–40 minutes following the addition of the serum from the side arm. Following this, the rate declined toward the control level, reaching this point in approximately 40 minutes. The rates shown in Table I represent average rates over the entire period of exposure (80 minutes) of the cells to the sera. Maximum rates ranged from 4 to 5 times control rates.   Serial dilution of the antiserum (in this case, the antiserum directed against antigen 10 was employed) progressively lowers the value of the maximum observed rate, as indicated in experiment 7, Table I. In experiment 5,

TABLE II

The effect of antisera against fertilizin on the respiratory rate of unfertilized
Strongylocentrotus purpuratus eggs

	Aver. rate (μl./min./vessel)	
Experiment No. 1 Antiserum from rabbit:		
d.	0.15	
e.	0.15	0.15 aver.
f.	0.15	
Pre-injection control serum:		
d.	0.14	
e.	0.14	0.14 aver.
f.	0.14	
Experiment no. 2 (trypsinized eggs) Antiserum from rabbit:		
d.	0.19	
e.	0.21	0.20 aver.
f.	0.20	
Pre-injection control serum:		
d.	0.16	
e.	0.17	0.16 aver.
f.	0.16	
Aver. rate, buffered sea water (12 determinations)	0.17 (Range—0.15–0.18)	

dealing with the effects of heterologous antisera directed against the *S. purpuratus* fertilizin antigen, a higher proportion of antiserum relative to the amount of egg suspension was employed in order to duplicate conditions existing during experiments with *S. purpuratus* eggs. The maximum rate observed with heterologous antisera was about three times the control rate. The high (ca. 7 times the control rate) rate observed with the homologous antiserum in this experiment presumably reflects the higher concentration of antibody employed.

As controls, some aliquots of eggs were exposed to sperm suspensions (0.1%) or to hypertonic medium (1 M final concentration with respect to NaCl) sea water, added from the side arm of the vessel. The increased respiration observed as a consequence of fertilization or treatment with hypertonic sea water approximates the

increases obtained using homologous antisera against fertilizin (Table I). Increased respiration following exposure to hypertonic medium was first reported by Warburg (1908), and confirmed by Keltch and Clowes (1947). The antiserum against extract of fertilized *Lytechinus pictus* eggs, washed and demembranated prior to homogenization, also proved effective in increasing the rate of respiration of *L. variegatus* eggs (results included in Table I).

Two preliminary experiments using unfertilized *S. purpuratus* eggs yielded less conclusive results. Slight increases in respiratory rate of questionable significance occurred after the addition of homologous antiserum against fertilizin. The increases in rate usually appeared within 30 minutes following addition of the sera, and lasted for 15–20 minutes, after which time the rates returned to the control level. Table II presents average rates during the time (60 minutes) the eggs were exposed to the sera. In experiment 2, the eggs were trypsinized as described above, on the assumption that a barrier exists at the egg surface which prevents combination of antibodies with the necessary sites on the egg. This treatment did not appreciably alter the results obtained during experiment 1. That the antisera against *S. purpuratus* fertilizin contained antibodies capable of increasing respiratory rate can be seen by the ability of these antisera to increase the respiration of *L. variegatus* eggs (Table I). In addition, these antisera were capable of blocking the first cleavage of demembranated fertilized *S. purpuratus* eggs, indicating the presence of antibodies against fertilizin (Tyler and Brookbank, 1956a, 1956b). Furthermore, positive ring precipitin tests were obtained with the homologous antigen. The problem of the failure of these antisera to cause an increase in the rate of respiration of the unfertilized eggs of *S. purpuratus* comparable to that observed using Lytechinus eggs remains unsolved at this writing. Since only two experiments are available, it may well be that future work will resolve the apparent difference between these two species.

Microscopic examination of Lytechinus eggs following these experiments revealed no morphological evidence of activation, excepting, of course, the cases in which the eggs had been inseminated. Samples of Lytechinus eggs treated with immune serum or hypertonic sea water in the manometer vessels were transferred to fresh sea water and observed periodically for 3 to 4 hours. No indications of membrane elevation or cleavage were seen in these eggs, though control eggs inseminated in the manometer vessels elevated fertilization membranes while in the vessels, and developed normally following transfer to fresh sea water. In some instances, Lytechinus eggs exposed to antisera against fertilizin were agglutinated (experiments 2, 3) and some darkening of the cortical region was noted. In most instances, the eggs tended to cytolyze in the antisera against fertilizin after 3–4 hours exposure.

Discussion

The above results indicate that antisera against fertilizin are capable of temporarily elevating the respiratory rate of unfertilized as well as fertilized (Tyler and Brookbank, 1956b) Lytechinus eggs. The time course followed by the increase to the maximum rate and the subsequent return to the control rate is similar in both cases. Judging from the increased maximum rate in the presence of a relatively greater amount of antiserum (Table I, experiment 5), it is probably safe to assume that the rate obtained in a given experiment is a function of antibody

concentration, provided the total volume and the number of eggs present remain constant. This is also indicated by experiment 7 (Table I), in which serial dilutions of antiserum against antigen number 10 were tested.

In comparing the results presented in this report with results obtained previously by others, it is apparent that increased respiratory rate in the presence of specific immune serum is seldom encountered, even over a rather wide range of biological material. The report by Sevag and Miller (1948) represents the only instance encountered by this author in which a temporary increase was observed. The increased respiratory rate was found only upon lysis of the *E. typhosa* cells in the presence of active complement, and was not observed when the cells were agglutinated. Complement was not added to the sera employed in the present study, nor were the sera heated to inactivate complement. Thus, the role of complement in the system causing the increased respiration of Lytechinus eggs is not known, though heating antisera against fertilizin to 56° C. for 30 minutes to inactivate C'1 and C'2 does not alter the cleavage blocking property of these antisera (Tyler and Brookbank, 1956a). The unfertilized eggs do not cytolyze during the period of measurement of respiratory rate, and remain intact for 3 to 4 hours following removal from the manometer vessels. After this time, a gradual cytolysis becomes evident. The increased oxygen consumption does not appear, therefore, to be associated with visible cytolytic changes in the egg, since the maximum respiratory rate in the presence of antisera against fertilizin is observed 20–40 minutes following the addition of the antisera from the side arm.

In considering the reports of Perlman (1954, 1957) and Perlman and Perlman (1957) on the parthenogenetic properties of antisera against extracts of unfertilized eggs and against fertilizin, one might be tempted to consider the increased rate of respiration of unfertilized eggs in the presence of antisera against fertilizin, or in hypertonic media, to be indicative of activation. This conclusion does not seem warranted by the data presented in this report. No morphological evidence of activation was encountered during the experiments, and one might more properly consider the increased respiration of eggs so treated to be analogous to the increases obtained in the presence of nitro-phenols (Clowes and Krahl, 1934, 1936; Krahl, 1950), methylene blue (Ballentine, 1940) and other substances which are not considered parthenogenetic. With regard to the failure to observe activation of eggs exposed to hypertonic sea water, it should be recalled that the time of exposure of unfertilized eggs to the proper hyper- or hypotonic medium is critical (Harvey, 1940). Therefore, failure to observe morphological signs of activation under the conditions prevailing in the manometer vessels is not surprising. In addition, a wide range of hyper- or hypotonic media are capable of eliciting increased respiration of the unfertilized eggs without effecting activation. The extent of the increases obtained depends on the degree of hyper- or hypotonicity employed, in much the same way as the extent of increases obtained with antisera against fertilizin depends on the amount of antibody present (Table III).

In conclusion, it seems appropriate to consider the purity of the fertilizin antigens used for injection. Special precaution was taken in the preparation of the *S. purpuratus* fertilizin antigen, and antigen number 10 (*L. variegatus* fertilizin), to insure minimum contamination with material from the eggs. These antigens were purified according to methods designed to yield electrophoretically homogene-

ous fertilizin, and were injected in exceedingly small amounts. The initial removal of fertilizin from the eggs was carried out at pH 3.5. Eggs treated at this pH for 2–3 minutes and returned to pH 8 develop normally, ruling against damage to the eggs by this degree of acidity. Antigen number 4 (*L. variegatus* fertilizin) was obtained in the same manner as number 10, except that the step involving precipitation of the antigen with ethanol was omitted. Results obtained using antisera against antigen number 10 paralleled those obtained using antisera against antigen number 4 completely. Apparently antisera against purified fertilizin are capable of temporarily raising the respiratory rate of the unfertilized egg. Results obtained using antiserum against extract of fertilized eggs parallel those obtained using antisera against fertilizin, since this antiserum also increased the rate of respiration of the unfertilized eggs. Since antibodies most probably cause their effects through combination with fertilizin at the egg surface, this last mentioned result seems to indicate the presence of fertilizin haptens in the fertilized-egg antigen. The most

TABLE III

The effect of varying degrees of hyper- and hypotonicity on the respiration of unfertilized Lytechinus variegatus eggs. Conditions the same as those prevailing for the experiments in Table I. (Hypertonicity in terms of excess NaCl, hypotoncity in terms of added distilled water)

Tonicity	Aver. rate μl./min./vessel
Experiment 1—hypertonic media	
2.9 × sea water	0.97
1.85 × sea water	0.55
Sea water	0.20
Experiment 2—hypotonic media	
0.84 × sea water	0.50
0.75 × sea water	0.58
0.66 × sea water	0.67
Sea water	0.28

probable location of these fertilizin haptens is the hyaline layer (ectoplasmic layer) of the fertilized egg, as proposed by Tyler and Brookbank (1956a).

SUMMARY

1. Homologous antisera against purified fertilizin, and against extract of fertilized eggs (of *Lytechinus pictus*) have been shown to temporarily increase the respiratory rate of the unfertilized eggs of *L. variegatus*. Parallel experiments employing antisera against fertilizin of *Strongylocentrotus purpuratus* and unfertilized *S. purpuratus* eggs yielded essentially negative results. Further experimentation is necessary before this apparent difference between the responses of the eggs of these two species to antisera against fertilizin can be resolved.

2. Antisera against fertilizin of *S. purpuratus* were effective in increasing the respiratory rate of unfertilized *L. variegatus* eggs, indicating the presence of antibody capable of effecting increased respiration.

3. Normal sera and antiserum against sperm were without measurable effect on the respiratory rate of *L. variegatus* eggs.

4. None of the eggs treated with antisera against fertilizin showed morphological evidence of activation.

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